Title

Hepatocyte-based *in vitro* model for assessment of drug-induced cholestasis

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Abstract

Early detection of drug-induced cholestasis remains a challenge during drug development. We have developed and validated a biorelevant sandwich-cultured hepatocytes- (SCH) based model that can identify compounds causing cholestasis by altering bile acid disposition. Human and rat SCH were exposed (24-48 h) to known cholestatic and/or hepatotoxic compounds, in presence or in absence of a concentrated mixture of bile acids (BAs). Urea assay was used to assess (compromised) hepatocyte functionality at the end of the incubations. The cholestatic potential of the compounds was expressed by calculating a drug-induced cholestasis index (DICI), reflecting the relative residual urea formation by hepatocytes co-incubated with BAs and test compound as compared to hepatocytes treated with test compound alone. Compounds with clinical reports of cholestasis, including cyclosporin A, troglitazone, chlorpromazine, bosentan, ticlopidine, ritonavir, and midecamycin showed enhanced toxicity in the presence of BAs (DICI $\leq$ 0.8) for at least one of the tested concentrations. In contrast, the in vitro toxicity of compounds causing hepatotoxicity by other mechanisms (including diclofenac, valproic acid, amiodarone and acetaminophen), remained unchanged in the presence of BAs. A safety margin (SM) for drug-induced cholestasis was calculated as the ratio of lowest in vitro concentration for which was DICI $\leq$ 0.8, to the reported mean peak therapeutic plasma concentration. SM values obtained in human SCH correlated well with reported % incidence of clinical drug-induced cholestasis, while no correlation was observed in rat SCH. This in vitro model enables early identification of drug candidates causing cholestasis by disturbed BA handling.

Keywords: drug-induced cholestasis; bile acids; sandwich-cultured hepatocytes; bile salt export pump; urea assay; safety margin

Abbreviations

ALT, alanine aminotransferase; ALP, aspartate aminotransferase; BAs, bile acids; BSEP/Bsep, bile salt export pump (human/rat); DICI, drug-induced cholestasis index; DMSO, dimethyl sulfoxide; TCA, taurocholic acid; CA, cholic acid; FBS, fetal bovine serum; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HBSS, Hanks' Balanced Salt Solution; NOAEL, no observed adverse effect level; NTCP/Ntcp, sodium taurocholate cotransporting polypeptide (human/rat); PBS, phosphate buffered saline; SCH, sandwich-cultured hepatocytes; SCRH, sandwich-cultured rat hepatocytes; SCHH, sandwich-cultured human hepatocytes; SM, safety margin; ULN, upper limit of normal.

Introduction

Cholestasis represents a pathological liver condition characterized by the impairment of bile secretion. Cholestasis is associated with accumulation of the bile acids (BAs) and other cholephiles in the liver (Fischer *et al.*, 1996). The cytotoxicity of accumulated BAs has been implicated as one of the major causes of hepatocellular damage noted during cholestasis (Attili *et al.*, 1986). BAs induce apoptosis at lower concentrations (in micromolar range), while they elicit necrotic damage to the cells at higher concentrations (in the milimolar range, close to the critical micelle concentration of BAs) (Perez and Briz, 2009).

BAs are synthesized from cholesterol in hepatocytes and their homeostasis is maintained by synchronized activity of different enzymes and transport proteins. The synthesized unconjugated BAs (e.g. cholic acid, chenodeoxycholic acid) are conjugated with either glycine or taurine and are excreted into the bile canaliculi by the bile salt export pump (BSEP/Bsep, *ABCB11/Abcb11*), an ATP-dependent efflux transporter. Conjugated and unconjugated BAs can further get sulfated or glucuronidated in the liver. Sulfated and glucuronidated BAs are transported into the bile canaliculi by multidrug resistance associated protein-2 (MRP2/Mrp2, *ABCC2/Abcc2*). Multidrug resistance protein-3 (MDR3/Mdr2, *ABCB4/Abcb4*) is a floppase that is involved in translocation of phosphatidylcholine from the inner to the outer bilayer of the bile canalicular membrane. In the bile duct, BAs form mixed micelles after associating with cholesterol and phophatidylcholine. The mixed micelles protect the bile duct surface from the detergent effects of the BAs (Elferink and Paulusma, 2007). In the intestine secondary BAs (deoxycholic acid, lithocholic acid, and ursodeoxycholic acid) are formed by the action of intestinal flora. BAs are taken up by the enterocytes via the apical sodium-dependent bile acid transporter (ASBT/Asbt; *SLC10A2/Slc10a2* gene). Heteromeric organic solute transporters OSTα-β, localized in the basolateral membrane of the enterocytes, effluxes the BAs to the portal circulation (Rao *et al.*, 2008; Dawson *et al.*, 2009). BAs are taken up from the portal blood into the hepatocytes by sodium taurocholate co-transporting polypeptide (NTCP/Ntcp; *SLC10A1/Slc10a1*) and by organic anion transporting polypeptides (OATP/Oatp; *SLCO/Slco*). The hepatic uptake of unconjugated BAs is mainly mediated by OATP1B1/Oatp1b2 (*SLCO1B1/Slco1b2*) while uptake of conjugated BAs is mostly carried out by NTCP (Dawson *et al.*, 2009; Xiang *et al.*, 2009; Csanaky *et al.*, 2011). Multidrug resistance associated protein-3 and -4 (MRP3/4 and Mrp3/4; *ABCC3/4 and Abcc3/4*) are the two transporters involved in basolateral (= sinusoidal) efflux of BAs. They are upregulated in cholestatic conditions, when the canalicular efflux of BAs is compromised (Bohan and Boyer, 2002; Alrefai and Gill, 2007).

Disturbances in the normal physiological function of the transporters and enzymes involved in BA homeostasis may lead to cholestasis. Altered enzyme/transporter function may originate from genetic mutations in transporters and enzymes and or external factors such as infections, inflammation, or physical obstruction of the common bile duct, as in the case of gall bladder stones (Epstein *et al.*, 1998; Wagner *et al.*, 2009). Depending on the underlying cause, distinction is made between intra- or extra-hepatic cholestasis. Progressive familial intrahepatic cholestasis (PFIC), benign recurrent intrahepatic cholestasis (BRIC), vanishing bile duct syndrome are some of the forms of intrahepatic cholestasis where modulation of functions of BSEP, MRP2, MDR3 are implicated (Pauli-Magnus and Meier, 2006). However cholestasis can occur from changes in a wide variety of proteins as listed previously (Balistreri *et al.*, 2005).

Increased risk of cholestasis has been reported with certain drug therapies (Stieger *et al.*, 2000). Drug-induced cholestasis has led to the market-withdrawal of troglitazone and nefazodone, while a safety warning has been added to the label of other drugs such as bosentan (Fattinger *et al.*, 2001; Funk *et al.*, 2001). Follow-up studies with these compounds have demonstrated (Marion *et al.*, 2007) inhibition of BSEP by these drugs, leading to intracellular accumulation of BAs and subsequent liver toxicity (Stieger *et al.*, 2000; Marion *et al.*, 2007). The putative role of disturbed BA homeostasis in different forms of hepatotoxicity has recently been substantiated by an untargeted metabolomics study with different hepatotoxicants. The study of Yamazaki et al., (2013) showed that the elevation of BAs in plasma and urine of rats is often one of the early events in drug-induced hepatotoxicity (Yamazaki *et al.*, 2013). The relationship between alteration in glycine-conjugated BA levels and the in vitro toxicity of exogenously administered primary BAs in SCH has been demonstrated as well (Chatterjee *et al.*, 2013).

Given the multiplicity and complexity of mechanisms underlying drug-induced cholestasis, early detection of corresponding safety issues during drug development remains highly challenging. Animal models of drug-induced cholestasis can provide valuable mechanistic insights about the progression of cholestatic diseases. However, the animal models suffer from several inherent differences with the human situation such as: (i) BA pools in humans and rodents are qualitatively and quantitatively distinct (Setchell *et al.*, 1997), (ii) the quantitatively major BAs in rodents (taurine conjugated) are more hydrophilic and less toxic than the major BAs present in human (glycine conjugated) (Rodriguez-Garay, 2003). Not surprisingly, with the current biochemical and histological markers only 50 % of the clinical cases of liver toxicity are detected in preclinical animal models (Olson *et al.*, 2000). In addition, the *in vitro* testing models using human hepatocytes detected only 50-60 % cases (drugs and drug candidates) of drug-induced liver injury (Xu *et al.*, 2008).

Existing *in vitro* models for detecting compounds which can cause cholestasis rely on determining the extent of inhibition of BSEP-mediated taurocholic acid (TCA) excretion in sandwich-cultured hepatocytes (SCH) (B-CLEAR®) (Marion *et al.*, 2007) or in BSEP/Bsep expressing vesicle models (Morgan *et al.*, 2010; Dawson *et al.*, 2012). These methods provide unique mechanistic information on potential interactions of drug candidate(s) with a representative BA (most often TCA) disposition in the liver. However, the limitations associated with these *in vitro* models are : (i) TCA is not a quantitatively important BA in human, and does not seem to play a significant role in hepatotoxicity upon its intracellular accumulation (Chatterjee *et al.*, 2013); (ii) the bioanalysis of TCA requires the use of a radiolabelled isotope or of LC-MS/MS instrumentation; (iii) multiple mechanisms are frequently involved in the toxicity exerted by a compound: even a mild inhibition of BSEP/Bsep can potentiate the existing toxicity due to concomitant reactive metabolite formation or direct mitochondrial toxicity by the compound (e.g. flutamide, ticlopidine, chlorpromazine) (Kang *et al.*, 2008; Anthërieu *et al.*, 2013; Yoshikado *et al.*, 2013); (iv) for some compounds e.g. troglitazone, the metabolite (troglitazone sulfate) is a more potent BSEP inhibitor than the parent compound (Funk *et al.*, 2001); direct *in vitro* BSEP inhibition studies with these compounds alone may not reveal the full implication of BSEP inhibition *in vivo*; (v) basolateral efflux of BAs in the hepatocytes becomes particularly important during hindrance in their canalicular efflux. For instance if a compound also inhibits MRP3/4,(apart from BSEP), BA accumulation at supra-physiological levels and subsequent bile acid-mediated liver injury is more likely to follow. It is noteworthy that inhibition of MRP4 has recently been shown to be associated with toxicity associated with certain HIV protease inhibitors (Fukuda *et al.*, 2013). SCH expressing the basolateral and canalicular transporters, provide us with the opportunity to investigate the effect of a xenobiotic on the overall disposition of BAs.

Clearly, evaluation of BSEP/Bsep inhibition is not sufficient to accurately predict drug-induced cholestasis for compounds exerting hepatotoxicity via multiple and/or complex mechanisms. This illustrates that there is an unmet need for a cost-effective, conceptually simple, higher-throughput in vitro model, granting reliable prediction of the liability of new drug candidates regarding drug-induced cholestasis.

We have developed a SCH-based *in vitro* assay to identify compounds that may cause cholestasis by interfering with BA disposition. The assay was validated using a set of known cholestatic (as positive control) and non-cholestatic but hepatotoxic compounds (as negative control) in both rat and human SCH. The clinical relevance of the assay was illustrated by demonstrating a correlation between *in vitro* cholestasis potential and clinical incidence data on cholestasis.

Material and Methods

Materials

Williams' E Medium (WEM), L-glutamine, penicillin-streptomycin mixture (contains 10,000 IU/ml potassium penicillin and 10,000 µg/ml of streptomycin sulfate), Dulbecco's modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Hanks' Balanced Salt Solution (HBSS) (referred to as 'standard buffer' when pH adjusted to 7.4), Phosphate Buffered Saline (PBS; 1x and 10x), and Trypan blue solution (0.4%) were purchased from Lonza Verviers SPRL (Verviers, Belgium). ITS+ ™ Premix (contains insulin 6.25 mg/l, transferin 6.25 mg/l, selenious acid 6.25 mg/l, bovine serum albumin 1.25 g/l and linoleic acid 5.35 mg/l) was purchased from BD Biosciences (Erembodegem, Belgium). Sulfuric acid (95-97%) was purchased from Chem-Lab NV (Zedelgem, Belgium). All BAs, collagenase type IV (from Clostridium histolyticum), ECM gel (from Engelbreth-Holm-Swarm murine sarcoma), recombinant human insulin, dexamethasone, urea, diacetyl monoxime, thiosemicarbazide, iron (III) chloride hexahydrate, ortho-phosphoric acid, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Dulbecco's modified Eagle's Medium 10x (DMEM 10x), and 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Cyclosporin A, troglitazone, and bosentan were purchased from Sequoia Research Products Ltd, UK. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from MP Biochemical (Illkirch, France). 48 and 24-well sterile cell culture plates were purchased from Greiner Bio-One BVBA (Wemmel, Belgium). Thermostable 96-well plates (for urea assay) were kindly provided by Greiner Bio-One BVBA (Wemmel, Belgium). Collagen was prepared in-house from rat tails according to established procedures. Erythromycin estolate, midecamycin, and troleandomycin were kindly provided by Prof. Erwin Adams (KU Leuven, Pharmaceutical Analysis).

Animals

The rats were housed in the Central Animal Facilities of KU Leuven, according to the guidelines and policies for animal experiments, housing and care, and the laws of Belgium and European Union. Studies were approved by the Institutional Ethical Committee for Animal Experimentation of KU Leuven. Rats were maintained in a 12 h light-dark cycle with free access to water and standard rat/mouse maintenance food (ssniff Spezialdiäten GmbH).

Isolation and culture of rat hepatocytes in sandwich configuration

Hepatocytes were isolated from male Wistar rats (170-200 g) based on a two-step collagenase perfusion method, as described previously, without adding trypsin inhibitor (Annaert and Brouwer, 2005). Rats were anaesthetized by an intraperitoneal injection of a mixture of xylazine (24 mg/kg) and ketamine (120 mg/kg). After isolation, cells were centrifuged (50 *g*) for 3 min at 4°C and the pellet was re-suspended in WEM containing 5 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Hepatocytes were counted using a hemocytometer and cell viability was determined using Trypan blue. Cells were re-suspended in WEM containing 5% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml insulin, and 1 µM dexamethasone (day-0 medium) and diluted to a final concentration of 1 x 10^6 cells/ml. Hepatocytes used in experiments had viability after isolation of at least 85%.

Rat hepatocytes were cultured in sandwich-configuration as previously described (Chatterjee *et al.*, 2013). Briefly, 24-well plates were coated with ice-cold neutralized collagen solution, and placed overnight at 37°C in a humidified incubator, and hydrated with PBS before use. Hepatocytes were seeded at a density of 0.5 x 10^6 cells/well, in 500 µl/well of day-0 medium. After incubating the cells at 37°C in a humidified atmosphere with 5% CO_2 (Binder CO_2 incubator, Binder GmbH) for 1-2 h, unattached cells were removed by shaking the plate and immediately aspirating the medium. To obtain a "sandwich" configuration, the cells were overlaid with 50 µl of rat tail collagen solution (~1.5 mg/ml, pH 7.4), (day-0). One hour later, pre-warmed day-0 medium was added onto the cultures which were kept in a humidified atmosphere with 5% CO_2. The medium was changed every day with culture medium consisting of WEM containing 1% (v/v) ITS+ ™ Premix, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1 µM dexamethasone (day-1 medium).

Sandwich-Cultured Human Hepatocytes

Human hepatocytes were isolated, cryopreserved and thawed as described previously (Alexandre *et al.*, 2012). The demographics and characteristics of the human hepatocyte batches used in the present study are listed in Table 1. 48-well sterile cell culture plates were coated one day before seeding with 50 µg/ml rigid collagen diluted in 0.02 N acetic acid (250 µl/well), and were placed overnight at 37°C in a humidified atmosphere with 5% CO_2. Immediately before seeding, the plates were washed twice with warm PBS and once with thawing medium. The thawing medium consisted of 10% (v/v) FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml insulin, and 1 µM dexamethasone in DMEM, while the seeding medium consisted of WEM, supplemented with the same additives as in the thawing medium. After thawing the cells in a waterbath (37°C), they were suspended in a mixture of 90 % Percoll® (diluted with with 10x PBS)

and thawing medium (1:1) at 37°C and centrifuged (168 g) for 20 min at room temperature. The pelleted cells were re-suspended in thawing medium and centrifuged again for 5 min (100 g) at room temperature. The pellet was then re-suspended in seeding medium and hepatocytes were counted using a hemocytometer and cell viability was determined using Trypan blue. The minimum viability obtained for all the batches was 90%. Cells were re-suspended in seeding medium and diluted to a final concentration of 1 x 10^6 cells/ml. The seeding density used was 0.2-0.25 x 10^6 viable cells/well, depending on the batch of hepatocytes. 24 h after seeding, the hepatocytes were overlaid with ECM gel solution (0.25mg/ml) in ice-cold WEM containing 1% (v/v) ITS+ ™ Premix, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1 µM dexamethasone. Medium was changed daily with day-1 medium.

Determination of urea formation in rat and human SCH

The capacity of the hepatocytes to convert ammonia to urea was used to assess the overall biochemical function and integrity of rat and human SCH. Urea formation by SCRH and SCHH was determined following protocols established in our lab (Chatterjee *et al.*, 2013). Briefly, the cells were incubated with HBSS containing 10 mM HEPES, 2 mM glutamine, 10 mM ammonium chloride and 3 mM ornithine (250 µl/well for 24-well plates; 125 µL/well for 48-well plates) for 1 h at 37°C in a humidified atmosphere with 5% CO_2. Next, 60 µl of the incubation medium/well was mixed with 240 µl of color reagent in a 96-well thermostable plate. The mixture of color reagent and incubate was heated at 85°C for 20 min in a water bath and subsequently cooled down by keeping the plate at 4°C for 10 min. Absorbance was measured at 525 nm using a Tecan Infinite M200 plate reader (Austria).

Fluorescence Microscopy

The biliary excretory function in rat and human SCH was assessed by qualitative evaluation of 5(6)-carboxy-2′,7′-dichlorofluorescein (CDF) excretion in bile canalicular networks via fluorescence microscopy (ex/em 490/520 nm), as previously explained (Wolf *et al.*, 2008). Briefly, day-5 SCHH and day-3 SCRH were rinsed with standard buffer and then incubated with standard buffer for 10 min at 37°C. Next, hepatocytes were incubated with 4 µM CDF diacetate (CDFDA) in standard buffer. After 10 min incubation at 37°C, the buffer was removed, hepatocytes were washed twice with standard buffer before fresh buffer was added. Hepatocytes and bile networks were imaged (both by fluorescence and light microscopy) with a VisiCam®3.0 camera (VWR International, Leuven, Belgium), mounted on an Olympus IX70 inverted tissue culture microscope (Olympus

Optical Co, GMBH, Hamburg, Germany). A monochromator (Polychrome IV; Till Photonics, Oberhausen, Germany) was used to generate the excitation wavelength (490 nm). For fluorescence microscopy a U-MWIB3 mirror unit was used (emission filter: 510 nm (long pass); dichroic mirror: 505 nm).

Incubations with BAs

Forty to sixty-fold concentrated solutions of a BA mixture (referred to as 40x and 60x BA mixture) consisting of the five quantitatively most important BAs present in the human plasma, were used (Gnewuch *et al.*, 2009; Scherer *et al.*, 2009; Xiang *et al.*, 2010). The 40x BA mixture consisted of 52.8 µM glycochenodeoxycholic acid (GCDCA), 15.6 µM of chenodeoxycholic acid (CDCA), 15.2 µM of glycodeoxycholic acid (GDCA), 16 µM of deoxycholic acid (DCA), and 14 µM of glycocholic acid (GCA); the 60x BA mix consisted of 79.2, 23.4, 22.8, 24 and 21 µM of GCDCA, CDCA, GDCA, DCA and GCA, respectively. Hepatocytes were first incubated with the test compounds alone for 2 h, to provide the test compound time to interfere with bile acid transporters. Subsequently, the test compound and the 40-60x BA mixtures (i.e. 60x for experiments with SCRH and 40x for experiments with SCHH) were co-incubated for 22 h. After the incubations, urea assay was performed for quantitative assessment of (compromised) hepatocytes functionality. For SCHH from two donors, hepatocytes were re-exposed a second time for 24 h to compound and BA mixture, followed by another urea assay, to assess the effect of re-exposure on in vitro cholestasis potency. All the BA solutions were prepared in day-1 medium.

Data Analysis

The Emax model was used to describe the concentration-dependency of the inhibitory effect of troglitazone, bosentan and chlorpromazine (Figure 1) with and without BA mixture, on the capacity of hepatocytes to produce urea:

$$E = Emax - \left[(Emax - Eo) \cdot \frac{C^n}{C^n + IC_{50}^n} \right] \qquad \text{(Eq. 1)}$$

Where E is the urea production by hepatocytes, E_{max} is the urea production under control condition (no compound was added), E_0 is the urea production at the maximum inhibitory effect of compound (and BA mixture), (E_{max} – E_0) is the maximum inhibitory effect. The IC_{50} is the compound concentration (with or without BA mixture) causing 50% inhibition of urea formation. The parameter "n" denotes the Hill factor. The best fits of the above equation to the individual urea formation data sets were obtained by non-linear regression analysis with the NLS package in R

version 2.15.1. The inverse of the experimentally obtained standard deviations were used for weighing.

To quantify the ability of a test compound to exert toxicity by disturbing BA homeostasis *in vitro*, a drug-induced cholestasis index (DICI) was calculated as follows:

$$DICI = \frac{Urea\ formation_{test\ compound+bile\ acids}}{Urea\ formation_{test\ compound\ alone}} \qquad \text{(Eq. 2)}$$

DICI values were calculated for each compound at every concentration examined. Compounds were classified empirically based on their DICI values: (i) compounds with mild or no potential to cause cholestasis: DICI > 0.8; (ii) compounds with moderate cholestasis risk: 0.8 ≥ DICI > 0.5 and (iii) compounds with high risk to cause cholestasis: DICI ≤ 0.5.

Safety margins were calculated for each compound, based on the Cmax (µM, mean peak plasma concentration in human, obtained from clinical reports) and the lowest *in vitro* concentration (µM) yielding a DICI < 0.8, as follows:

$$Safety\ Margin\ (SM) = \frac{lowest\ concentration\ (\mu M) yielding\ DICI \leq 0.8}{Cmax\ (\mu M)} \qquad \text{(Eq. 3)}$$

When DICI was > 0.8 for the highest concentration tested, a minimum safety margin was reported (SM > highest concentration/C_{max} (µM)). For the test compounds evaluated, the concentration range included between the Cmax and Cmax/10 was considered to be the therapeutic plasma concentration range (see Figures 2 and 3).

Statistics

For troglitazone (Figure 1), the ANOVA (F-test) in MS Excel version 2007 was used to evaluate statistical significance of differences between urea formation profiles by SCRH treated with troglitazone alone and with troglitazone plus the 60x BA mixture: separately fit profiles for troglitazone alone or for troglitazone plus BAs were compared to the simultaneous fit obtained with both data sets combined . For each concentration of chlorpromazine and bosentan (Figure 1), a two-tailed student t-test was used to assess the statistical significance of the differences in urea formation (nmol/well) between SCRH treated with compounds alone or SCRH treated with compound plus 60x BA mixture. The criterion for statistical significance was $p < 0.05$.

Results

Influence of BAs on concentration-dependent toxicity of bosentan, troglitazone and chlorpromazine in SCRH: proof of concept

The working hypothesis of the present study was that SCH would be sensitized to the cytotoxic effects of BAs upon co-incubation with compounds reported to cause cholestasis in the clinic. To test this hypothesis, SCRH were exposed to increasing concentrations of bosentan, chlorpromazine and troglitazone with or without a 60x BA mixture. For bosentan concentrations > 100 μM, SCRH became sensitive to the toxic effects of BAs. This was reflected by decreased urea formation in SCRH co-incubated with bosentan and BA mixture as compared to bosentan or BA mixture alone (Figure 1A).

Similarly, SCRH were also sensitized to the cytotoxic effects of BAs when co-incubating the cholestatic compounds chlorpromazine and troglitazone with the 60x BA mixture (Figure 1B-C). In case of chlorpromazine, the difference in urea formation between SCRH treated with chlorpromazine alone and SCRH co-incubated with chlorpromazine and BA mixture was only observed at the highest concentration (30 μM) of chlorpromazine treatment (Figure 1B). However, for troglitazone, co-incubation with the BA mixture resulted in decreased urea formation for all concentrations tested (except for 50 μM). The concentration-dependent urea formation profiles for troglitazone with and without 60x BA mixture were significantly different from each other ($p < 0.05$).

The effect of BAs in modulating in vitro cytotoxicity of the cholestatic compounds is further illustrated in Table 2. The IC_{50} for reduction of urea formation by the compounds in the presence of the BA mixture is consistently lower than when SCRH were treated with compounds alone. The concentration-dependent decrease in DICI values (supplementary Table 1) is consistent with the cholestatic nature of these compounds.

Effect of co-incubation with a BA mixture on toxicity of known cholestatic compounds in different batches of SCRH

To determine the effect of the 60x BA mixture on the concentration-dependent toxicity of known cholestatic compounds in multiple batches of SCRH, cyclosporin A (n = 6 batches), troglitazone (n = 3), bosentan (n = 1), chlorpromazine (n = 2), ritonavir (n = 2) and glyburide (n = 3), were incubated with and without BA mixture (Figure 2). Mean (±SEM) DICI values (Eq. 2) were determined for each compound following the urea formation measurements in three wells with and three wells without BAs. DICI values obtained from incubations with cyclosporin A at ≥ 10 μM and for troglitazone at ≥ 75 μM in different batches of SCRH were lower than 0.8. For bosentan,

chlorpromazine and glyburide, an increasing number of DICI values were found to be lower than 0.8, as the concentration of the incubated compounds increased. In contrast, DICI values remained > 0.8 for ritonavir at all the concentrations tested (1-200 μM).

For each compound a safety margin (SM) was obtained (Eq. 3) based on the ratio of lowest in vitro concentration for which DICI ≤ 0.8, to the reported plasma Cmax. SM values ranged between 5.6 for cyclosporin A to 244 for glyburide. For ritonavir a SM > 13.1 was reported since the highest evaluated in vitro concentration yielded a DICI > 0.8 (Table 3).

Effect of co-incubation with a BA mixture on toxicity of known cholestatic compounds in different batches of SCHH

The mean (±SEM) DICI values shown in Figure 3A-B were determined following incubations with and without 40x BA mixture in SCHH obtained from various donors (demographics in Table 1) at different concentrations of cyclosporin A (n = 6 batches), troglitazone (n = 4), bosentan (n = 4), chlorpromazine (n = 2), ritonavir (n = 2), ticlopidine (n = 1), midecamycin (n = 3), rosiglitazone (n = 1), erythromycin estolate (n = 1) and troleandomycin (n = 2). Consistent with the results in SCRH, experiments with cyclosporin A at ≥ 10 μM and troglitazone ≥ 75 μM produced DICI values below 0.8. Incubations with 1 μM of bosentan in one out of four SCHH batches (lot number S240908) yielded a DICI lower than 0.8. For chlorpromazine, midecamycin, ticlopidine (100 μM), ritonavir, DICI values were observed to go below 0.8 at more than one concentration. However, for rosiglitazone, erythromycin estolate and troleandomycin, none of the DICI values were ≤ 0.8.
Similar to SCRH, a SM value was calculated for compounds in SCHH. SM values varied between 0.2 in case of bosentan to 15.6 for troglitazone (Table 3). Compounds with SM < 30 are considered to show significant risk to cause cholestasis in the clinic (see discussion for details).

Effect of co-incubation with a BA mixture on toxicity of negative control compounds in different batches of SCRH and SCHH

Compounds that mediate hepatotoxicity by mechanisms other than interfering with BA homeostasis, along with a known non-hepatotoxicant (warfarin) were selected as negative control compounds. Diclofenac and valproic acid were used as negative control compounds in SCRH (Figure 4), yielding DICI values > 0.9 for all the concentrations tested. In SCHH, mean (±SEM) DICI values determined for 20 μM amiodarone, 1 mM acetaminophen and 500 μM warfarin were always > 0.90 (Table 4).

Effect of repeated exposure of SCHH to compound and BA mixture on in vitro estimation of cholestasis potential

The influence of repeated exposure to compounds and the 40x BA mixture on the toxicity of bosentan, midecamycin, ritonavir and troleandomycin was evaluated in two batches of SCHH (Table 5). Following the urea assay after 24 h incubation, the same cells were re-exposed to the same concentration of compound and BA mixture for another 24 h. The DICI values were determined both after 24 and 48 h of co-incubation. Consistently lower DICI values were obtained after 48 h co-incubation compared to 24 h co-incubation. For ritonavir a DICI $\leq$ 0.8 was obtained after re-exposure (2 incubation periods of 24 h, Table 5), which contrasted to the unchanged DICI following single exposure.

Correlation between safety margin in SCHH and clinical incidences of cholestasis

To correlate the in vitro estimated cholestasis potential of known cholestatic compounds in the current assay (in SCHH) with their clinically-reported incidence data for cholestasis, safety margins were calculated (Eq. 3). The incidences of hepatotoxicity were obtained from literature (Ticktin and Zimmerman, 1962; Klintmalm *et al.*, 1981; Larrey and Erlinger, 1988; Naschitz *et al.*, 1995; Sulkowski, 2004; Humbert *et al.*, 2007; Dhillon and Keating, 2009). As no literature incidence data were available for midecamycin, it was not included in this correlation analysis. For troleandomycin and ritonavir DICI values obtained after 24 h exposure remained > 0.8 at all concentrations, therefore minimum SM values were used for plotting. Two separate correlation analyses were carried out (one with and one without bosentan): bosentan yielded a marginally decreased DICI value ($\leq$ 0.8, but standard deviation overlapping with 0.8 cut-off) for a low concentration only (no concentration-dependent decrease in DICI). Compounds yielding a safety margin > 30 (rosiglitazone, erythromycin estolate) were not included in the analysis. For cyclosporin A and bosentan, the reports from two independent literature sources were combined (weighed to the number of patients in the literature reports), while for ritonavir a range was obtained in the literature (the mean of the range was used for correlation). No particular clinical marker for cholestasis was selected, as the individual reports employed different markers of general hepatotoxicity. Figure 5 illustrates that clinical incidences of cholestasis decrease with increasing calculated safety margin for cyclosporin A, bosentan, troglitazone, ritonavir, chlorpromazine and ticlopidin. A linear correlation (r^2=0.85; r^2 = 0.86, when bosentan included) was obtained between safety margin and incidence (%) of cholestasis.

Discussion

At the hepatic level, xenobiotics can disturb BA homeostasis, by interfering with any of the following stages of BA disposition: (i) uptake of BAs from the basolateral side of the hepatocytes, (ii) de-novo synthesis of the BAs (iii) metabolism/conjugation inside the hepatocytes, (iv) efflux of

BAs to the bile canaliculi via canalicular transporters, and (v) sinusoidal efflux of BAs via basolateral efflux transporters. Consequently, a holistic *in vitro* model that covers the various stages of hepatic BA disposition would be best equipped to accurately predict cholestatic potential of a drug (or drug candidate) altering intrahepatic BA homeostasis. Hepatocytes in sandwich-culture have been shown to preserve the functions of proteins controlling the different stages of BA disposition (Chatterjee *et al.*, 2013; De Bruyn *et al.*, 2013). We have presently developed a SCH based assay that can classify drugs/compounds based on their potential to cause cholestasis in the clinic via altered BA homeostasis. In vitro biliary excretory capacity of the SCHH used in the present study was qualitatively verified at day-5 by measuring MRP2-mediated CDF efflux into bile canaliculi (as an in-process control, supplementary Figure 1).

For the purpose of the present study we have composed a BA mixture, containing five BAs based on their quantitative importance in human plasma, as reported previously (Gnewuch *et al.*, 2009; Scherer *et al.*, 2009; Xiang *et al.*, 2010). The toxicity exerted by the compounds (alone or in combination with the BA mixture) was measured by the urea assay, which is a marker for integrity of liver specific-function (Chatterjee *et al.*, 2013). The 40x-60x concentrated BA mixtures selected for the present study are higher than the physiological BA levels in plasma, but become relevant in case of cholestasis. Hepatic BA concentrations have been reported to reach 430 – 800 μM in case of cholestasis (Fischer *et al.*, 1996; Rolo *et al.*, 2003).

The concentrations of the BA mixtures were selected such that they would not affect the urea formation by the cultures when incubated alone. In contrast, co-incubation of the same cultures in the presence of the BA mixture and increasing concentrations of a cholestatic compound should lead to toxicity (decreased urea formation). In other words, it was hypothesized that the presence of BAs in the extracellular medium would sensitize the hepatocytes towards the cholestatic action of the compounds interfering with BA homeostasis. An increased toxicity in the presence of the BA mixture with 100-200 μM of bosentan, 30 μM of chlorpromazine and with 75-150 μM of troglitazone, clearly vindicate this hypothesis (Figure 1). This suggests that at those concentrations, the hepatocytes showed decreased ability to dispose off the added BAs, consistent with the cholestatic action of the compounds.

Based on the design of this new in vitro model as well as the results obtained, we introduce the concept: “drug-induced cholestasis index” (DICI). DICI is a relative measure for the residual urea formation when a cholestatic drug is incubated in the presence of BAs as compared to the urea formation when the drug is applied separately. DICI values classify compounds according to their ability to potentiate the *in vitro* cytotoxicity of the BAs in hepatocytes, and this is expected to be related to the potential of compounds to cause cholestasis in vivo. The DICI value measurements in

human hepatocytes are particularly relevant for clinical conditions, as they may provide indication of the cholestatic signature of the compound in the clinic. The interbatch variability of the human hepatocytes regarding increasing the toxicity of cholestatic compounds in the presence of BAs mixture may reflect to some extent the variability in toxic response of these compounds in the clinic. For instance, a DICI of 0.66 ± 0.08 was obtained with 20 μM cyclosporin A in batch S0906A, while in S240908 a DICI of 0.21 ± 0.12 was obtained with 15 μM of cyclosporin A treatment. A similar interbatch variability was noted for troglitazone, ritonavir, bosentan among other compounds. These findings support the use of multiple batches of SCHH to achieve adequate sensitivity for detecting cholestatic drug candidates.

Cyclosporin A and troglitazone are two known cholestatic compounds that elicited a clear concentration-dependent decrease of DICI in different batches of SCRH and SCHH (Figure 2 and 3). In addition, the concentration-dependent decrease of DICI was also observed for bosentan, chlorpromazine and troglitazone in rat hepatocytes (Figure 1, Supplementary Table 1). The concentration dependency of the DICI values strengthens the hypothesis that disturbances in BA homeostasis mediated by the compounds, contributes to the cholestatic effect.

Rosiglitazone is an analogue of troglitazone with a decreased risk of hepatotoxicity. With troglitazone treatment, 1.9 % of the patients had ALT > 3 times upper limit of normal (ULN), as opposed to only 0.17 % patients with rosiglitazone (Lebovitz *et al.*, 2002). However, previous reports suggested a strong BSEP inhibition potential of rosiglitazone (Dawson *et al.*, 2012). When troglitazone and rosiglitazone were evaluated for cholestasis potential with our assay in the same batch of human hepatocytes, troglitazone yielded a DICI ~ 0, while the DICI value for rosiglitazone was 1.2 at the same concentration (100 μM). The higher DICI for rosiglitazone compared to troglitazone illustrates that the assay is competent to differentiate between cholestatic and non-cholestatic compounds with similar chemical motifs.

DICI values in the range 1.5-2.0 were obtained for glyburide and ritonavir in SCRH. These compounds are reported to interfere with the transporters taking part in BA uptake (Leslie *et al.*, 2007). At high concentrations, these compounds might prevent the BAs to enter into the hepatocytes, thus decreasing the intracellular accumulation and in turn displaying a protective effect as illustrated by DICI values exceeding 1. These findings suggest further investigation regarding a more sequential incubation design to minimize interference between BAs and cholestatic compounds at the hepatic uptake level.

Further to DICI, corresponding safety margin (SM) values were calculated. The SM reflects the ratio of the lowest concentration of the compound that yields a DICI ≤ 0.8 to the mean peak plasma concentration that the compound (drug) reaches in the clinic (C_{max}). To account for different

sensitivity and/or distinct intracellular accumulation and metabolism as applicable in vitro *versus* in vivo, a SM cut-off value of 30 was used to classify compounds as cholestatic *versus* non-cholestatic. Thus a compound with a SM < 30 is considered to show significant cholestasis risk in the clinic. A SM cut-off value of 30 has been suggested previously for predicting clinical toxicity via in vitro models, e.g. for cardiotoxicity (Yao *et al.*, 2008). The rationale for employing this safety margin towards compound decisions can be further illustrated by the case of cyclosporin A, which has been reported to accumulate in the liver several folds more than in the plasma (Lacerda *et al.*, 1995). Consistently, when the pharmacokinetic profile of cyclosporin A was simulated in SimCYP (Version 12, release 1, Sheffield, UK), the hepatic Cmax was found to be 7.6 times higher than plasma Cmax (supplementary Figure 2). While hepatocyte accumulation of cyclosporin A is likely to also occur in SCH, the actual accumulation ratio may be different due to distinct transporter and metabolizing enzyme expression profiles between in vitro and in vivo. Such in vitro-in vivo discrepancies necessitate the use of a safety margin to support reliable compound decisions. In addition, recent reports (Dawson *et al.*, 2012; Anthërieu *et al.*, 2013) suggest that hepatotoxicity manifested by a compound is contributed by different toxicity-pathways acting simultaneously. Again, the safety margin will help to compensate for in vitro-in vivo discrepancies in the quantitative role of these pathways.

For troglitazone, chlorpromazine and ticlopidine, multifaceted events have been reported for their toxic outcome. Reactive metabolite formation has been described for troglitazone along with BSEP inhibition (He *et al.*, 2004). In addition, the metabolite troglitazone-sulfate has been reported to be a much stronger inhibitor of BSEP compared to the parent compound (Funk *et al.*, 2001). The toxicity of another drug, chlorpromazine, with 2-5 % incidence of cholestasis (Larrey and Erlinger, 1988; Lewis and Zimmerman, 1999; Parmentier *et al.*, 2013), has been attributed to increased oxidative stress, inherent mitochondrial toxicity of the compound along with BSEP inhibition and consequent BA accumulation (Anthërieu *et al.*, 2013). In contrast, it has not shown strong BSEP inhibition in any of the prior *in vitro* reports (Morgan *et al.*, 2010; Dawson *et al.*, 2012). Oxidative stress in addition to the inhibition of other transporters apart from Bsep, such as Mdr3 and Mrp2 have been associated with ticlopidine-induced cholestasis (Yoshikado *et al.*, 2013). Previously applied *in vitro* assays using vesicles (Dawson *et al.*, 2012) have shown mild inhibition of hBSEP and rBsep with IC_{50} values of 74 μM and 49 μM, respectively. These concentrations are much higher than the clinically relevant free drug concentrations (Dawson *et al.*, 2012). The vesicles/BSEP over-expressed cell lines lack the metabolic machineries required to produce the clinically relevant metabolites exhibiting the oxidative stress. On top of that, they lack the cellular organelles like mitochondria, which can be a direct target for many drugs. Therefore, the final clinical toxicity (that

can be contributed by toxic insults arising from multiple pathways, and not only by the direct inhibition of BSEP-mediated BA transport by the parent drug) remains poorly detected in current drug evaluation programs. In the presently developed assay clinically relevant SM values of 15.6, 10.6 and 12.4 were obtained for troglitazone, chlorpromazine and ticlopidine in SCHH, thus flagging the three drugs for their cholestasis risk.

A linear correlation was obtained between reported clinical cholestasis incidences (%) of test compounds and their corresponding SM in experiments conducted with human SCH (Figure 5). As the SM values of the compounds increase the clinical cholestasis incidence (%) decreases. Incidence values based on ALP increase > 2 times upper limit of normal, ULN (with or without ALT > 3 times ULN), which has been described as a prominent sign of cholestatic liver injury and mixed hepatocellular injury, was preferred whenever available in literature (Navarro and Senior, 2006; Aithal *et al.*, 2011). The clinical symptomatic difference between cholestasis and other forms of hepatotoxicity is not always indicated in literature for the compounds examined here. Therefore hepatotoxicity incidence values that were used for bosentan and troglitazone have reported ALT > 3 time ULN as hepatotoxicity marker (Humbert *et al.*, 2007). Note that midecamycin could not be included in the correlation analysis in Figure 5 as clinical cholestasis incidence data could not be obtained for it. However, midecamycin has been reported to cause cholestasis, consistent with a SM value of 10 in our in vitro assay. This was in contrast with previously reported in vitro BSEP/Bsep inhibition potential, yielding a high Ki value (154 μM) (Horikawa *et al.*, 2003).

Regarding the correlation shown in Figure 5 as well as the more qualitative classification represented in Figure 6, the in vitro data were unambiguous for the majority of the test compounds. However, further investigation is warranted for bosentan, ritonavir, troleandomycin, and erythromycin estolate. The latter represents the only misclassified compound by our in vitro assay. In SCHH, the SM for erythromycin was > 69, while the DICI value appeared to increase with increasing concentration. This again suggests possible interference between BAs and erythromycin at the level of hepatic uptake, as also mentioned above for glyburide and ritonavir in rat hepatocytes. Moreover, a higher inhibitory effect on the uptake of BAs has been suggested for erythromycin estolate in SCRH, which will further decrease the accumulation and hence the expected increased toxicity in presence of BAs may not be evident (Ansede *et al.*, 2010). In addition, erythromycin has been shown to accumulate in the liver: 150-fold higher hepatic concentrations than serum concentrations have been reported for erythromycin in rats (Lee *et al.*, 1953). Although the uptake transporters are qualitatively maintained in SCH, a down-regulation of uptake transporters with culture time has been reported (Tchaparian *et al.*, 2011). This may decrease

the uptake of the drug and hence intracellular accumulation compared to the in vivo situation. Investigation with higher concentrations of erythromycin can be suggested to tackle the issue.

Ritonavir did not yield a DICI ≤ 0.8 after 24 h co-incubation, with the current assay design. This resulted in a "minimum" safety margin (> 6.6), meaning that this HIV protease inhibitor could not be unambiguously classified as safe (SM cut-off 30) based on the present in vitro data. Consequently, the minimum safety margin was used to correlate to incidence (%) of hepatotoxicity in Figure 5. The higher hepatotoxicity incidences with ritonavir may be attributed to the fact that the patients treated with ritonavir have already become susceptible to liver injury due to the existing HIV infection. In addition to that, ritonavir is often co-administered with other anti-HIV drugs such as atazanavir which potentiates the cholestasis incidences in the clinic (Rakotondravelo *et al.*, 2012). The safety margin calculated here is obtained with human hepatocytes that are not infected with HIV, neither exposed to other drugs. A "minimum" safety margin was also employed for the antibiotic troleandomycin (SM > 10 in SCHH), which could thus also not be classified as non-cholestatic. This is consistent with reports of cholestatic jaundice (Ticktin and Zimmerman, 1962) warranting further investigation with a higher concentration and possibly different incubation design.

Although bosentan did show a DICI ≤ 0.8 at 1 μM in SCHH (DICI = 0.75 ± 0.14), there was no evidence of a concentration-dependent increase in cholestasis potential. In contrast, bosentan did show concentration-dependent increase in cholestatic potential in SCRH (Figure 1 and 2). In this context, it is noteworthy that bosentan has more potent interaction with rat Ntcp compared to human NTCP (Leslie *et al.*, 2007). In addition, following re-exposure of 200 μM bosentan and BA mixture to SCHH, the DICI value obtained was ≤ 0.8 (DICI = 0.75 ± 0.16). Taken together this *in vitro* assay classifies bosentan as a compound with a potential cholestasis risk, but requiring further investigation (Figure 5 and 6). These findings may explain the differential and complex interplay of bosentan with rat and human transporters responsible for BA disposition. Figure 5 illustrates that SM values correlated well with hepatotoxicity incidence percentages, irrespective of whether bosentan was included (dotted line) or excluded (solid line).

Repeated exposure to compounds and BA mixture was examined to improve the sensitivity of the in vitro cholestasis model (Table 5). The non-destructive nature of urea assay provided the opportunity to re-expose the cells with compound and BA mixture, after one urea assay has been carried out. As mentioned above, 100 μM ritonavir produced a DICI ≤ 0.8 after repeated (but not single) exposure. Also for cyclosporin A, troglitazone, bosentan, midecamycin and troleandomycin, DICI values tended to decrease after repeated exposure.

The negative control compounds were selected such that they are known to cause hepatotoxicity (diclofenac, valproic acid, amiodarone, acetaminophen), but by mechanisms other than interaction with BA homeostasis, along with a non-hepatotoxicant warfarin. None of the negative control compounds produced a DICI value ≤ 0.8 in both SCHH and SCRH. Diclofenac, acetaminophen and amiodarone have been reported to cause hepatotoxicity by reactive metabolite formation while for valproic acid, the intrinsic toxicity coupled with a reactive acyl glucuronide moiety have been implicated in its hepatotoxicity (Bort *et al.*, 1999; Manyike *et al.*, 2000; Kiang *et al.*, 2011; Zahno *et al.*, 2011). The test results with negative control compounds suggest that the current assay will not result in increased in vitro toxicity of compounds in presence of BA mixture, if the compounds do not interfere with BA disposition in vivo.

Our data warn for ignoring species differences in drug toxicity. The previously reported in vivo toxicity of chlorpromazine and troglitazone in rat could not explain the observed clinical toxicity. For chlorpromazine, only moderate increase (< 2 times) of aspartate amino transferase (AST) and bilirubin levels have been reported in rat (Schoonen *et al.*, 2007). Another study reported no significant increase in the levels of bilirubin or ALP, however a three times increase of γ-glutamyl transpeptidase was reported (Obata, 1983). In addition, the in vitro BSEP/Bsep inhibition studies could not distinguish between the difference in hepatotoxic sensitivity of rat and human towards chlorpromazine. Similar hBSEP and rBsep inhibition IC_{50} values (147μM in human versus 122μM in rat) were reported for chlorpromazine (Dawson *et al.*, 2012). However, in the current model a > 3 times higher SM was obtained for chlorpromazine in rat compared to human, thus corroborating the species-specific cholestatic/hepatotoxic response observed. Also the animal models with currently available biomarkers could not detect the cholestatic /hepatotoxic nature of troglitazone in rat (Li *et al.*, 2002; Marra *et al.*, 2005), although strong Bsep/BSEP inhibition was observed in vitro (Dawson *et al.*, 2012). Consistently, the in vitro data presently obtained with our cholestasis assay resulted in similar SM values in rat and human SCH (Table 3). Thus, the in vitro cholestasis assay could overcome the inability of the present animal models to detect cholestatic nature of drugs; in addition it can also indicate the species-specific sensitivity towards cholestatic action of the compounds.

This model supports the use of sandwich-cultured human (but not rat) hepatocytes as preclinical model, as the correlation between SM and incidence is only observed in case of SCHH, but not in SCRH.

In conclusion, we have established a new *in vitro* model based on sandwich-cultured hepatocytes, to assess the potential of a compound to cause cholestasis by disturbing BA homeostasis. The model employs toxicity (reduced urea formation capacity) as an endpoint and has been validated with positive and negative control compounds in human SCH. With SCHH, 8 out of 9 known cholestatic

compounds (with previous reports of disturbing BA homeostasis (Figure 6)) were flagged. Ritonavir, bosentan and troleandomycin were identified as compounds with potential cholestasis risk requiring further investigation. There were no compounds that were incorrectly flagged for cholestasis. We have shown with compounds like chlorpromazine, troglitazone, ticlopidine, that if a compound requires metabolism along with BA disposition disturbances to exert its toxicity, the model is competent to identify it as a potentially cholestatic compound. This model can give new insights into the toxicity mechanisms associated with different hepatotoxicants disturbing BA homeostasis. It carries the promise to decrease the use of laboratory animals for preclinical testing of drug-induced cholestasis.

Figure legends

Figure 1A-C: Concentration-dependent effect of bosentan (A), chlorpromazine (B) and troglitazone (C) on urea formation capacity of day-4 sandwich-cultured rat hepatocytes (SCRH), in presence (Δ) and absence (○) of a bile acid (BA) mixture. SCRH were pre-incubated for 2 h with the test compound followed by 22 h co-incubation with a 60x BA mixture and the test compound. Subsequently the urea formation by day-4 SCRH was measured. Each data point represents mean (±SEM) urea formation from three wells. The fitted curves were obtained as described in the methods section. For bosentan and chlorpromazine alone, toxicity was not observed for a sufficient number of concentrations to obtain a fitted curve, therefore no IC_{50} values were calculated. The concentration-dependent urea formation profiles of troglitazone with and without 60x BA mixture were significantly different ($p < 0.05$, denoted by *) from each other. The urea formation with 60x BA mixture were significantly different ($p < 0.05$ denoted by *, student t-test) from the urea formation with compounds alone treatment, only at the highest concentrations for bosentan and chlorpromazine.

Figure 2: Drug-induced cholestasis index (DICI) and safety margin (SM) values of different concentrations of cyclosporin A, troglitazone, glyburide, ritonavir, chlorpromazine, and bosentan in SCRH. Points represent mean (±SEM) DICI values obtained in different batches of SCRH. Unique batches of SCRH are designated by the same symbols across different panels. The compounds were incubated with and without BA mix (three wells with and three wells without BA mix), as described in the methods section, and DICI values were calculated based on the relative urea formation. The dotted line on the Y-axis represents a DICI value of 0.8, while the dotted line on the X-axis represents the reported Cmax of each compound. The shaded area covers DICI values below 0.8 (i.e. flagged for cholestasis risk) for concentrations within the therapeutic plasma concentration range (between Cmax/10 and Cmax).

Figure 3A-B: DICI values (at various concentrations) and SM values obtained in SCHH with cyclosporin A, troglitazone, bosentan, chlorpromazine, ritonavir, ticlopidine, midecamycin, rosiglitazone, erythromycin estolate and troleandomycin. Points represent mean (±SEM) DICI values obtained with human hepatocytes from different donors (symbols refer to unique donors across different panels; see Table 1 for donor information). DICI values were calculated based on the urea assay following 24 h co-incubation of the compounds with or without BA mixture (three wells with and three wells without BA mix), as described in the methods section. A DICI value of 0.8 is indicated by the dotted line on the Y-axis, while the dotted line on the X-axis represents the

reported Cmax for each compound. The shaded area covers DICI values below 0.8 (i.e. flagged for cholestasis risk) for concentrations within the therapeutic plasma concentration range (between Cmax/10 and Cmax).

Figure 4: Mean (±SEM) DICI (▼) values (at different concentrations) as well as SM values for diclofenac and valproic acid in SCRH. The dashed line on the Y-axis represents a DICI value of 0.8, while a dotted line on the X-axis represents Cmax of the respective compound. The shaded area covers DICI values below 0.8 (i.e. flagged for cholestasis risk) for concentrations within the therapeutic plasma concentration range (between Cmax/10 and Cmax).

Figure 5: Correlation between literature reports of incidences of cholestasis (%) and SM values (r^2=0.85) in SCHH, for cyclosporin A, troglitazone, ritonavir, chlorpromazine, ticlopidine and troleandomycin. The dotted line represents the correlation (r^2 =0.86) including bosentan (■), which yielded borderline DICI values (≤ 0.8) two times in SCHH (see discussion). Incidences were obtained from clinical reports of hepatotoxicity of these drugs. The mean incidence from two different reports was used for both cyclosporin A and bosentan (weighteded for the number of patients). For chlorpromazine and ritonavir the reported range of cholestasis incidence is shown. Compounds with a SM > 30 were not included in the analysis (see discussion). Troleandomycin and ritonavir (●) did not yield DICI values ≤ 0.8, however the minimum SM values based on the highest concentration tested in vitro were below 30. For midecamycin no literature reports of incidence of cholestasis (%) were available, hence it was not included in this correlation analysis.

Figure 6: Classification of compounds based on the *in vivo* clinical cholestasis report and *in vitro* cholestatic potential with the current assay in SCHH. Compounds that have both reports of clinical hepatotoxicity/cholestasis and reports of BAs homeostasis disturbance (*in vitro* or *in vivo*) are classified in + segments on the Y-axis, while absence of any of these characteristics classifies them in – segments on the Y-axis. Compounds that have yielded a SM < 30 are classified in the + segment on the X-axis, while compounds with SM > 30 in the – segments on the X-axis. The shaded area (top segment) represents compounds that have reports of both clinical hepatotoxicity/cholestasis and BAs homeostasis disturbance, but could not be classified as safe with a SM > 30 (their minimal SM fell below 30 based on DICI at highest concentration tested).

Conflict of Interest Statement

There are no conflicts of interest.

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Web reference 2: http://livertox.nih.gov/Ticlopidine.htm

Table 1: The demographics and batch characteristics of the cryopreserved human hepatocyte batches (donors) used in this study. * indicates that batch SC1034 was a freshly plated human hepatocyte batch. Bile canaliculi formation was evaluated (see supplementary Figure 1) based on CDF excretion capacity of the SCHH, with fluorescence microscopy. The symbols for the human hepatocytes batches used in Figure 3A and 3B are also represented.

Lot number (symbol)	Gender	Race	Age (years)	Viability (%)	Yield (million/vial)	Bile canaliculi function
B0403VT (O)	Female	Caucasian	47	90	9	Yes
SC1034* (▲)	Male	Caucasian	58	78	(NA)	Yes
S240908 (■)	Female	Caucasian	49	93	9	Yes
S0312VT (Φ)	Male	Caucasian	58	92	11	Yes
S0906A (Π)	Female	Caucasian	72	92	7.5	Yes
S1108VT (Δ)	Female	Caucasian	41	90	9	Yes
S1409A (◐)	Female	Caucasian	75	90	8	Yes
S0212A (♦)	Male	Caucasian	61	92	5	Yes
S2203LT (⊙)	(NA)	(NA)	(NA)	92	12	Yes

NA = Not available

Table 2: IC_{50} ± SD (μM) for bosentan, chlorpromazine and troglitazone with and without 60x BA mixture in SCRH. The IC_{50} values were obtained as described in the methods section. For bosentan and chlorpromazine incubated alone, toxicity was not observed for a sufficient number of concentrations to obtain a fitted curve. Therefore no IC_{50} values were calculated, instead the minimum IC_{50} (μM) values (from highest concentration tested) are reported.

	With 60x BA	Compound alone
Bosentan	135 ± 5.5	> 200
Chlorpromazine	25.0 ± 1.8	> 30

Troglitazone	92.3 ± 3.8	126 ± 0.2

Table 3: Safety margins (SM) for different model drugs evaluated in both SCHH and SCRH evaluated in the present study. The safety margins were calculated as described in the methods section.

Compound name	SCRH	SCHH
Bosentan	13.5	0.14
Ritonavir	> 13.1	> 6.60
Cyclosporin A	5.60	8.30
Midecamycin	(ND)	10.2
Troleandomycin	(ND)	> 10.2
Chlorpromazine	31.9	10.6
Ticlopidine	(ND)	12.4
Troglitazone	11.7	15.6
Erythromycin estolate	(ND)	> 68.7
Rosiglitazone	(ND)	> 96.2
Glyburide	244	(ND)

ND= Not determined

Table 4: Mean (± SEM) DICI values of amiodarone, acetaminophen and warfarin in SCHH.

Compound	Concentration (μM)	Lot number	DICI ±SEM
Acetaminophen	1000	S1108VT	0.93±0. 34
Amiodarone	20	SC1034	1.24±0. 05
Warfarin	500	S0212A	1.31±0. 19

Table 5: Effect of repeated exposure of SCHH to compound and BA mixture on drug-induced cholestasis index (DICI) values (mean ± SD). Cyclosporin A and troglitazone were evaluated in the same batch batch, while other compounds in a different batch. The values in bold show DICI ≤ 0.80.

	24 h co-incubation	48 h co-incubation
Bosentan 1 μM	1.11 ± 0.09	1.02 ± 0.13
Bosentan 5 μM	0.98 ± 0.03	0.85 ± 0.07
Bosentan 10 μM	1.03 ± 0.09	0.98 ± 0.11
Bosentan 200 μM	0.85 ± 0.07	**0.75 ± 0.16**
Cyclosporin A 15 μM	**0.74 ± 0.15**	**0.46 ± 0.18**
Midecamycin 100 μM	1.00 ± 0.19	1.07 ± 0.07
Ritonavir 1 μM	0.95 ± 0.09	0.92 ± 0.12
Ritonavir 50 μM	1.02 ± 0.12	0.92 ± 0.08
Ritonavir 100 μM	0.99 ± 0.08	**0.80 ± 0.39**
Troglitazone 100 μM	**0.66 ± 0.13**	**0.61 ± 0.09**
Troleandomycin 10 μM	1.00 ± 0.09	0.83 ± 0.26
Troleandomycin 20 μM	1.12 ± 0.13	0.91 ± 0.08

Table 6: Incidence (%) of hepatotoxicity, clinical hepatotoxicity marker, number of patients used in the respective study and reported human BSEP inhibition IC_{50} (μM) for the model cholestatic drugs used in our study.

Drug	hBSEP inhibition IC_{50} (μM)	Incidence (%)	Clinical markers	Patient population	Reference
Bosentan	38.1	7.2	ALT[d] > 3 ULN[b]	4623 pulmonary arterial hypertension patients	(Dawson *et al.* 2012; Humbert *et al.* 2007)

		11	ALT > 3 ULN	658 pulmonary arterial hypertension patients	(Dhillon and Keating 2009)
Chlorpromazine	147.6	2-5	ALT > 3 ULN		(Dawson *et al.* 2012; Larrey and Erlinger 1988)
Cyclosporin A	0.5	19.7	Bilirubin>2 ULN	66 renal transplant patients	(Dawson *et al.* 2012; Klintmalm *et al.* 1981)
		4.4	Elevation of liver enzymes and bilirubin	705 kidney transplant, 112 heart transplant, 75 liver transplant	Web reference 1
Erythromycin estolate	4.1	2	ALP[e] > 3 ULN		(Dawson *et al.* 2012; Lewis and Zimmerman 1999)
Ritonavir	2.2	5.3-9.5	ALT, AST > 3 ULN	1270 infected patients	(Morgan *et al.* 2010; Sulkowski 2004)
Rosiglitazone	6.4	0.17	ALT> 3 ULLR	3503 diabetic patients	(Dawson *et al.* 2012; Lebovitz *et al.* 2002)
Ticlopidine	74	4	ALP > 3 ULN		(Dawson *et al.* 2012); Web reference 2
Troglitazone	2.7	1.9	ALT> 3 ULLR[c]	2510 diabetic patients	(Dawson *et al.* 2012; Lebovitz *et al.* 2002)
Troleandomycin	(ND)	4	ALP >3 ULN	50	(Ticktin and Zimmerman 1962)

[b]Upper limit of normal; [c]Upper limit of reference range; [d]Alanine aminotransferase; [e]Alkaline phosphatase

Figure 1

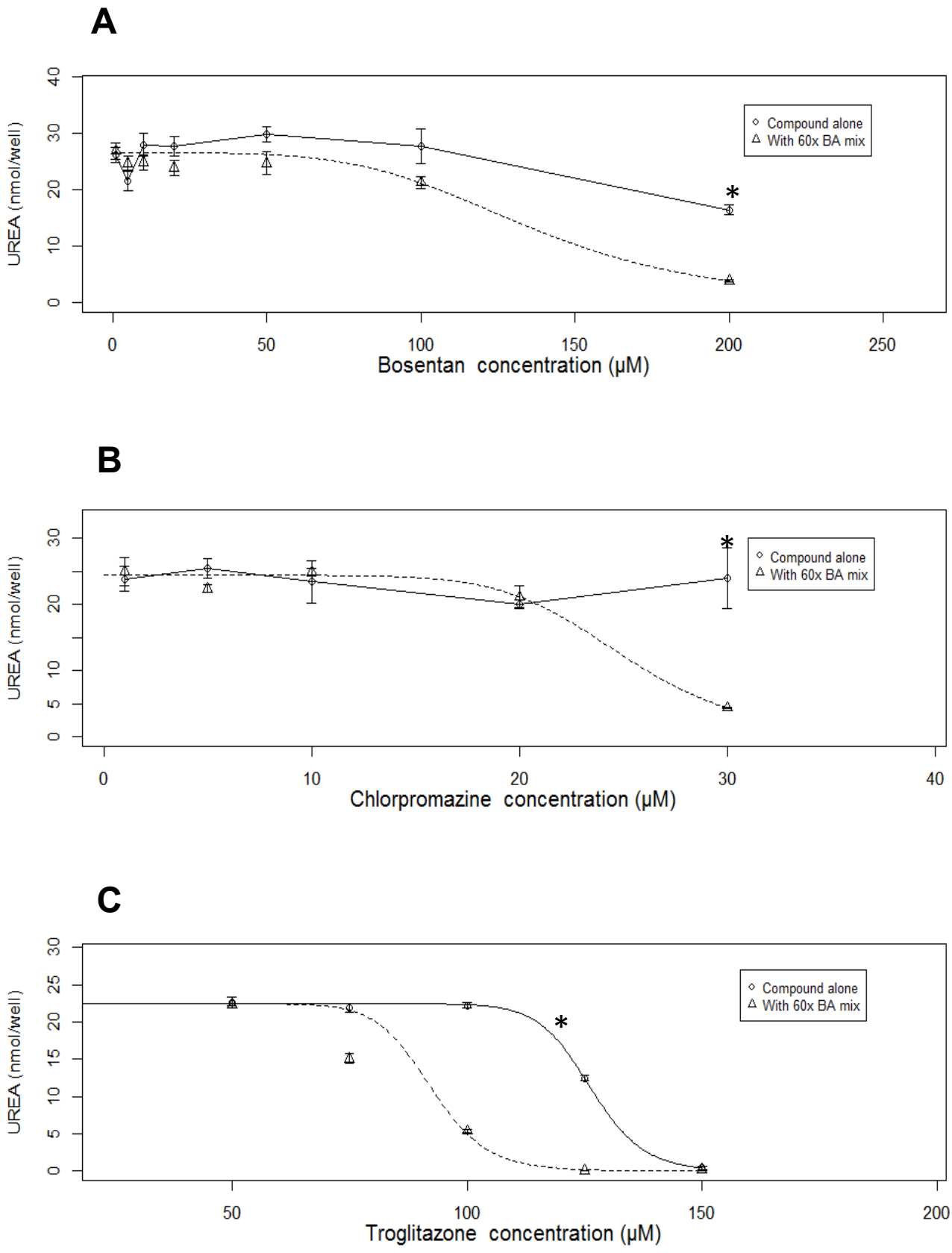
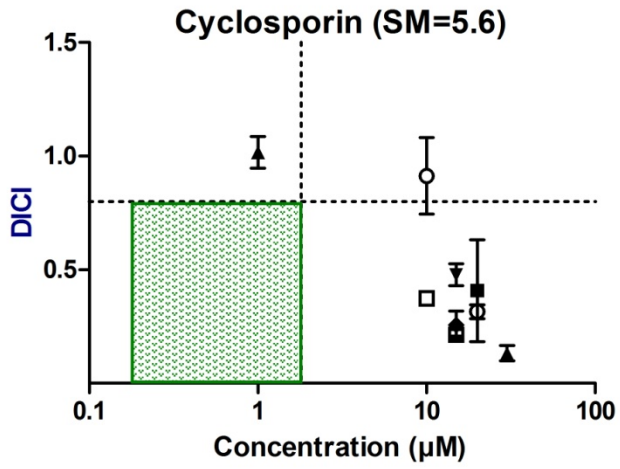
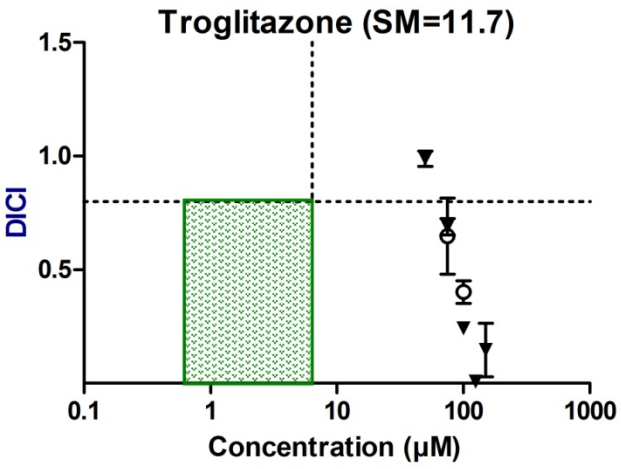
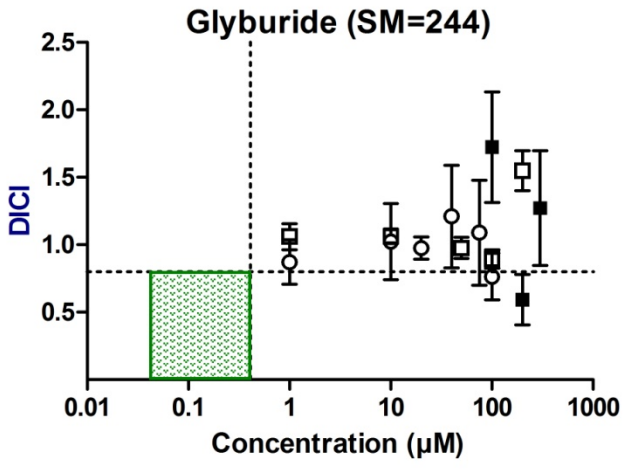
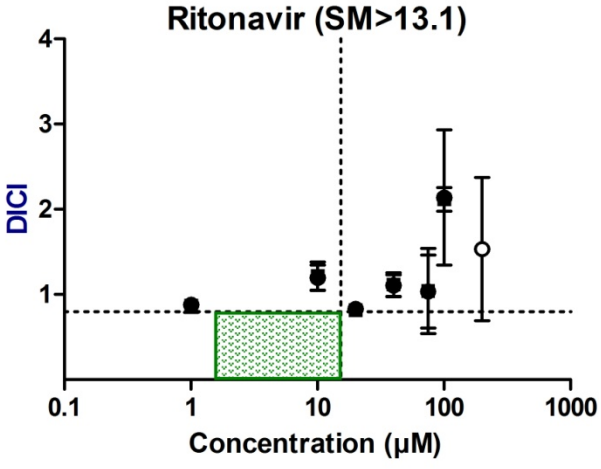

A
UREA (nmol/well)
0
10
20
30
40
0
50
100
150
200
250
Bosentan concentration (μM)
Compound alone
With 60x BA mix
*
B
UREA (nmol/well)
0
5
10
20
30
0
10
20
30
40
Chlorpromazine concentration (μM)
Compound alone
With 60x BA mix
*
C
UREA (nmol/well)
0
5
10
15
20
25
30
50
100
150
200
Troglitazone concentration (μM)
Compound alone
With 60x BA mix
*

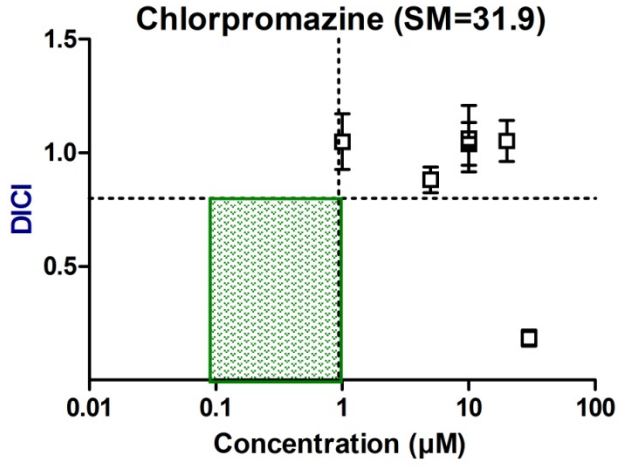
Figure 2


Cyclosporin (SM=5.6)
DICI
1.5
1.0
0.5
0.1
1
10
100
Concentration (μM)


Troglitazone (SM=11.7)
DICI
1.5
1.0
0.5
0.1
1
10
100
1000
Concentration (μM)


Glyburide (SM=244)
DICI
2.5
2.0
1.5
1.0
0.5
0.01
0.1
1
10
100
1000
Concentration (μM)


Ritonavir (SM>13.1)
DICI
4
3
2
1
0.1
1
10
100
1000
Concentration (μM)


Chlorpromazine (SM=31.9)
DICI
1.5
1.0
0.5
0.01
0.1
1
10
100
Concentration (μM)

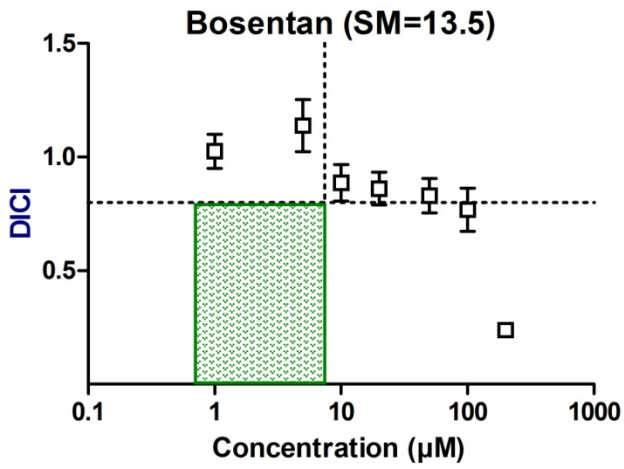

Bosentan (SM=13.5)
DICI
1.5
1.0
0.5
0.1
1
10
100
1000
Concentration (μM)

Figure 3A

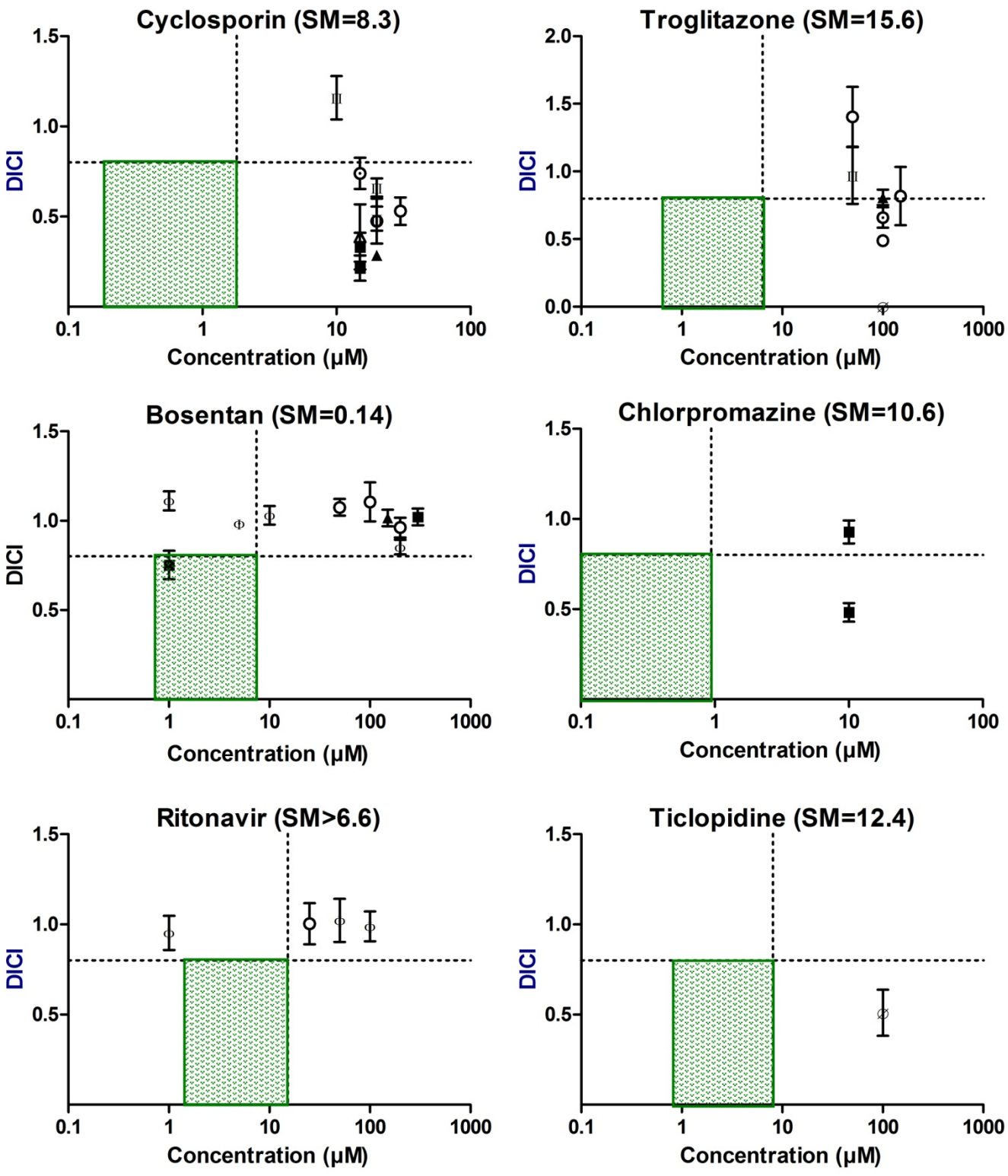
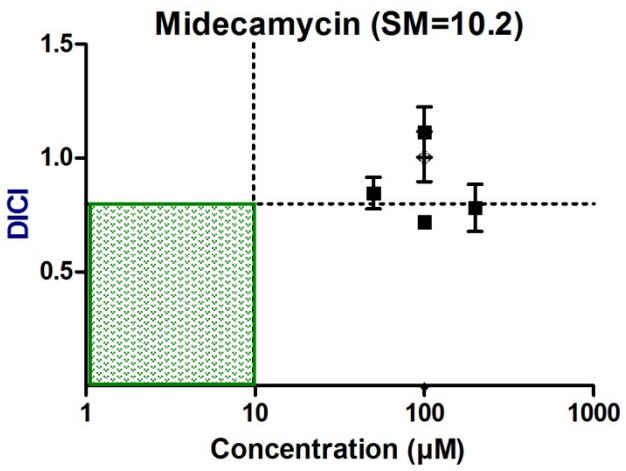
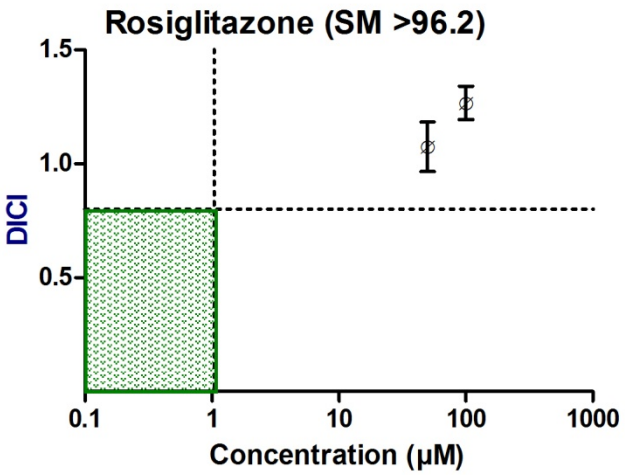
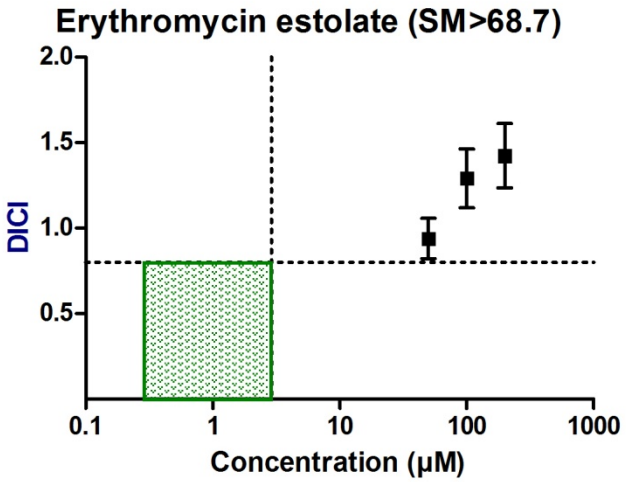

Cyclosporin (SM=8.3)
Troglitazone (SM=15.6)
Bosentan (SM=0.14)
Chlorpromazine (SM=10.6)
Ritonavir (SM>6.6)
Ticlopidine (SM=12.4)
DICI
Concentration (μM)

Figure 3B


Midecamycin (SM=10.2)
DICI
1.5
1.0
0.5
1
10
100
1000
Concentration (μM)


Rosiglitazone (SM >96.2)
DICI
1.5
1.0
0.5
0.1
1
10
100
1000
Concentration (μM)


Erythromycin estolate (SM>68.7)
DICI
2.0
1.5
1.0
0.5
0.1
1
10
100
1000
Concentration (μM)

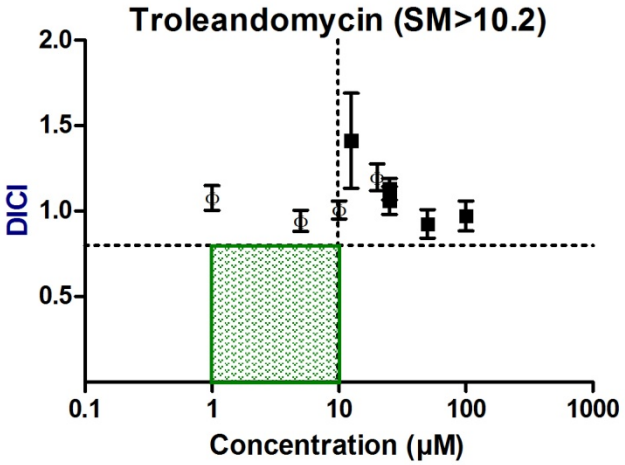

Troleandomycin (SM>10.2)
DICI
2.0
1.5
1.0
0.5
0.1
1
10
100
1000
Concentration (μM)

Figure 4

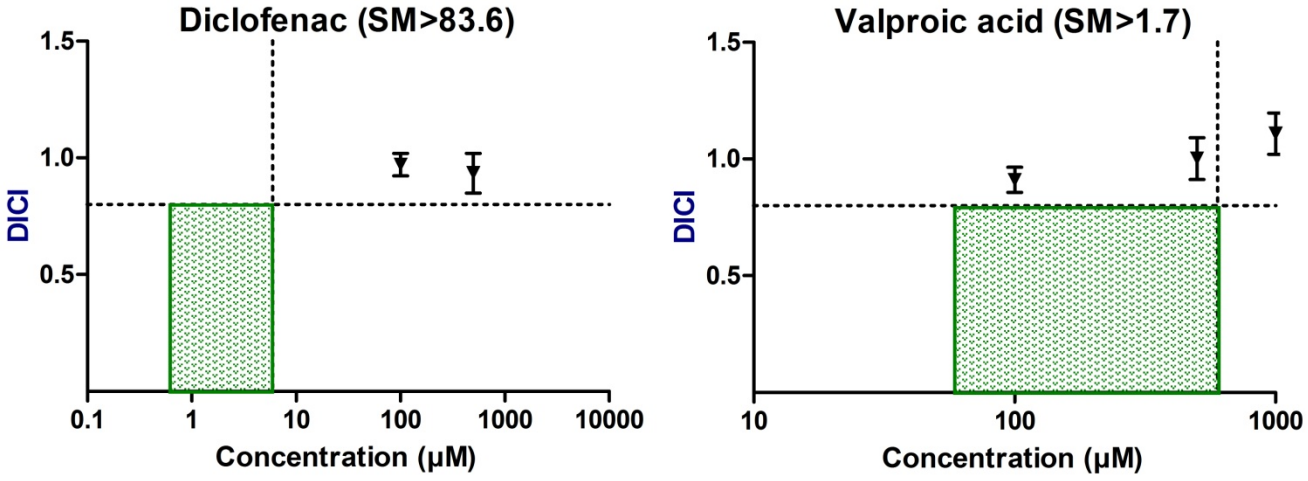
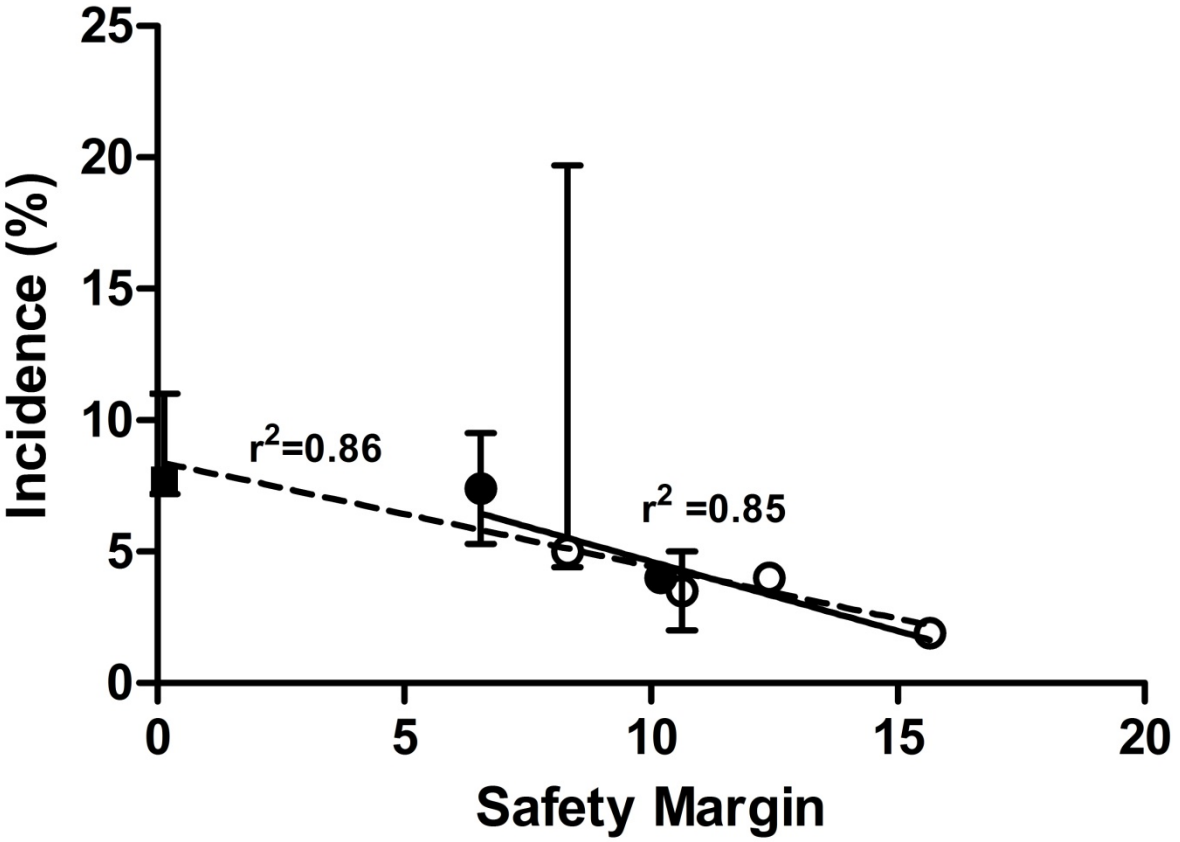
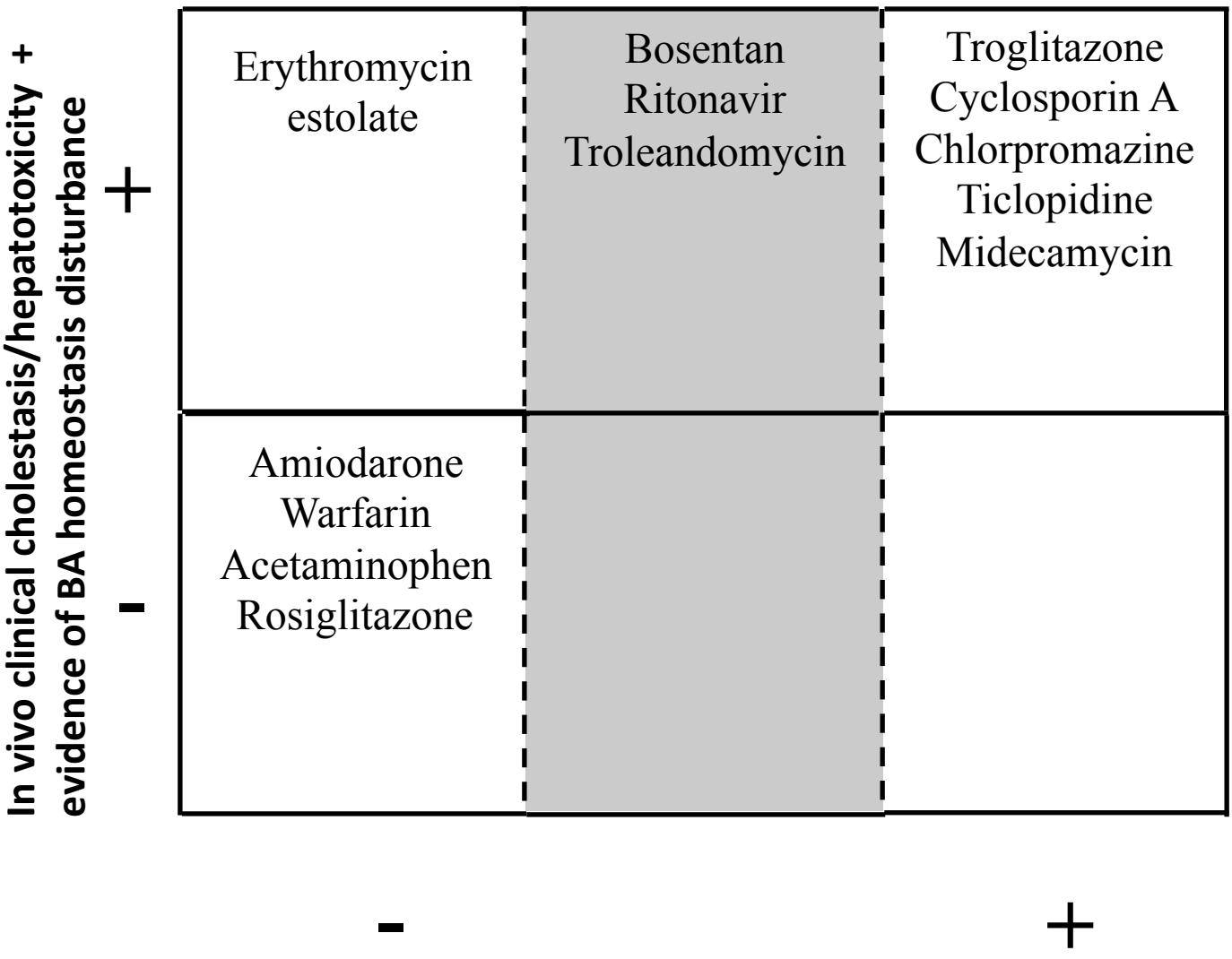

Diclofenac (SM>83.6)
DICI
1.5
1.0
0.5
0.1
1
10
100
1000
10000
Concentration (µM)
Valproic acid (SM>1.7)
DICI
1.5
1.0
0.5
10
100
1000
Concentration (µM)

Figure 5




Erythromycin estolate
Bosentan
Ritonavir
Troleandomycin
Troglitazone
Cyclosporin A
Chlorpromazine
Ticlopidine
Midecamycin
Amiodarone
Warfarin
Acetaminophen
Rosiglitazone
In vivo clinical cholestasis/hepatotoxicity + evidence of BA homeostasis disturbance
+
-
-
+
In vitro cholestasis risk with SM < 30